Paragraph beginning at page 1, line 9:

The present invention relates to a biosynthetic gene cluster of *Micromonospora echinospora* spp. *calichensis*. In particular, the calicheamicin biosynthetic gene cluster contains genes coding for proteins and enzymes used in the biosynthetic pathway and construction of calicheamicin's aryltetrasaccharide and aglycone, and the gene conferring calicheamicin resistance. The present invention also relates to isolated genes of the biosynthetic cluster and their corresponding proteins. In addition, the invention relates to DNA hybridizing with the calicheamicin gene cluster and the isolated genes of that cluster. The invention also relates to expression vectors containing the biosynthetic gene cluster, the individual genes, or functional variants thereof.

Paragraph beginning at page 6, line 3

Calicheamicin's molecular architecture in conjunction with its useful biological activity and potential therapeutic value brand calicheamicin a target for the study of natural product biosynthesis. While the radical-based mechanism of oxidative DNA cleavage by calicheamicin (i.e. aromatization of the bicyclo[7.3.1]tridecadiynene core structure, via a 1,4-dehydrobenzene-diradical, resulting in the site specific oxidative double strand DNA cleavage) is well understood, it was unknown, prior to this invention, how *Micromonospora* constructs calicheamicin. As a result, before the present invention, there was a need to discover and understand calicheamicin biosynthesis. Prior to this discovery of the present

inventors, knowledge of genes coding for nonchromoprotein enediyne biosynthesis was completely lacking.

Paragraph beginning at page 6, line 13

The toxicity of the enediyne compounds, including calicheamicin, centers on the problem of directing the compound to cleave only the DNA of interest, such as tumor cell DNA, and not the DNA of the host. Due to calicheamicin's powerful ability to cleave DNA, scientists have investigated the mechanism by which calicheamicin-producing organism protects itself against the DNA-cleaving activity of the molecule (Rothstein, D. M., *Enediyne Antibiotics as Antitumor Agents,* p. 77 (1995)). Prior to this invention, knowledge of genes coding for non-chromoprotein enediyne self resistance was completely lacking.

Paragraph beginning at page 25, line 15

The second screening was based on the assumption that calicheamicin's biosynthetic cluster would also contain genes coding for deoxysugar ligand synthesis. Further, it was postulated that all hexopyranosyl ligands of calicheamicin diverged from the common intermediate 4-keto-6-deoxy TDP-D-glucose (30), Figure 5, as macromolecule-sugar synthesis in many organisms began with a similar common intermediate. Thus, it was believed that the cluster coding for calicheamicin biosynthesis, in addition to carrying a PKS-encoding region, would carry both a common glucose-l-phosphate nucleotidyltransferase and a NDP-α-D-glucose 4,6-dehydratase gene, encoding the putative enzymes E_{p1}, and E_{od}, respectively. See figure 5. These enzymes are necessary to convert a sugar (12)(figure 5) to the hypothesized common intermediate, 4-keto-6-deoxy TDP-D-glucose (30). Analogs to

4,6-dehydratases have been previously characterized from *E. coli, Salmonella,* and *Streptomyces.* Additionally, a nucleotide transferase from *Salmonella* has been characterized as an α-D-glucose-1-phosphate thymidylyltransferase. The secondary screen was performed using a probe based upon the postulation that the *M. echinospora*'s calicheamicin synthesis would begin from a similar precursor found in *E. coli, Streptomyces* and *Salmonella,* and that this precursor required a dehydratase to convert it into the common intermediate, 4-keto-6-deoxy TDP-D-glucose (30). In particular, a DNA probe (designated E_{od}^{I}) was designed from the conserved NAD^{+}-binding site of bacterial NDP-α-D-glucose 4,6-dehydratases. He, X., et al., *Biochem., 35,* 4721-4731 (1996). Southern hybridization of the genomic *M. echinospora* cosmid library with the E_{od}^{I} probe revealed cross-hybridization with clones 4b, 10a, 13a, 56, and 60. Two additional clones, designated 58 and 66, were also identified in this screen. See Figure 1. This secondary hybridization indicated the clustering of genes encoding both polyketide and deoxysugar biosynthesis.

B4 cont.

Paragraph beginning at page 27, line 5

The clones positive for PKS I and II and deoxy sugar biosynthesis homology and calicheamicin resistance were used to map the biosynthetic cluster. Southern hybridization established similarity between clones 3a, 4a, 4b, 10a, 13a, 16a and 56. In addition, nucleotide sequence overlaps were found between clones 4b, 13a, and 56. See Figure 1. Restriction mapping and Southern hybridization of these clones indicated that the positive cosmid clones corresponded to a continuous region of the *M. echinospora* chromosome spanning > 100 kb. The present invention thus provides for cosmids having a nucleic acid molecule from *Micromonospora echinospora* coding for a nonchromoprotein enediyne biosynthetic cluster.

B5

Paragraph beginning at page 28, line 20

The *cal*C locus was isolated by identifying calicheamicin genomic cosmid clones that were able to grow on Luria Bertani ("LB") agar plates containing ampicillin and calicheamicin. The DNA of the positive clones (clones that grew on the plates containing calicheamicin) was isolated and subsequent restriction mapping localized the desired phenotype (calicheamicin resistance). The DNA was then sequenced and the open reading frames analyzed to ascertain the orf coding for the desired phenotype. *In vitro* studies were also performed and confirmed the ability of CalC to inhibit DNA cleavage.

Paragraph beginning at page 29, line 4

DNA containing *cal*C was cloned into an inducible vector, using known methods, resulting in overexpression of *cal*C. The polypeptide product (CalC) was then isolated and purified to homogeneity. Analysis of the purified CalC revealed that it is a non-heme iron metalloprotein that functions via inhibition of calicheamicin-induced DNA cleavage *in vitro*. Another aspect of the invention is an expression vector containing *cal*C or a fragment of *cal*C coding for a bioactive molecule. There is also provided a transformed host cell, preferably bacteria, more preferably *E. coli*, containing *cal*C or a fragment of *cal*C coding for a bioactive molecule. Such transgenic expression of *calC* results in an 10^5-fold increase in calicheamicin resistance in *E.coli*, a 100-fold increase in resistance in *S.lividans*, and a 50-fold increase in resistance in yeast.

Paragraph beginning at page 35, line 5

One aspect of the invention relates to an isolated DNA strand containing the *cal*G gene and having the DNA sequence SEQ ID. NO.: 5. Another aspect of the invention is the protein, CalG, having amino acid sequence SEQ ID. No.: 6. According to BLAST analysis, *cal*G encodes a 4,6-dehydratase. Dehydratases had been characterized from *E. coli, Salmonella* and *Streptomyces,* (Thompson, M. et al., *J. Gen. Microbiol.*, 138, 779-786 (1992); Vara, J.A., et al., *J. Biol. Chem., 263,* 14992-14995 (1988)), and analogous NDP-D-glucose 4,6-dehydratases had been characterized from a variety of organisms. Liu, H.-w., et al., *Ann. Rev. Microbiol., 48,* 223-256 (1994); Hallis, T.M., et al., *Acc. Chem. Res.,* in press (1999). Based upon these prior studies, it was known that the overall transformation catalyzed by 4,6-dehydratases is an intramolecular oxidation-reduction where an enzyme-bound NAD^+ receives the 4-H as a hydride in the oxidative half-reaction and passes the reducing equivalents to C-6 of the dehydration product in the reductive half-reaction. Thus, it appears that Cal G is necessary for the formation of the aryltetrasaccharide 4,6-dideoxy-4-hydroxylamino-D-glucose moiety. CalG appears to be a TDP-D-glucose 4,6-dehydratase which catalyzes the conversion of intermediate 13 into intermediate 30. (See figure 5). Another aspect of the invention is an expression vector containing *cal*G or a fragment of *cal*G encoding for a bioactive molecule. There is also provided a transformed host cell, preferably bacteria, more preferably, *E. coli*, containing *cal*G or a fragment of *cal*G encoding for a bioactive molecule.

Paragraph beginning at page 36, line 6

There is also disclosed an isolated DNA strand containing the *cal*S gene. Based on sequence homology with other P450-oxidases, CalS appears to be a P450-oxidase homolog which performs the oxidation of intermediate 39 to intermediate 42

(figure 5). The oxidation may occur at the nucleotide sugar level or hydroxylamine formation after the sugar has been transferred to the aglycone. There is also provided an expression vector containing the *cal*S gene or a fragment of *cal*S coding for a bioactive molecule. There is also provided a transformed host cell, preferably bacteria, more preferably *E. coli*, containing *cal*G or a fragment of *cal*G coding for a bioactive molecule.

B9 cancel

Paragraph beginning at page 36, line 22

B10

There is also provided an expression vector containing the *calQ* gene or a fragment of *calQ* coding for a bioactive molecule. There is also provided a transformed host cell, preferably bacteria, more preferably *E. coli*, containing *calQ* or a fragment of *calQ* coding for a bioactive molecule.

IN THE CLAIMS:

Please cancel claims 1-8, 57, 58, 97, 100, 101, 142-144, 146, 148, and 149 without prejudice.

Please amend the claims as follows:

B11

9. (twice amended) An isolated nucleic acid molecule, wherein said nucleic acid molecule comprises SEQ ID No. 35.

B12

88. (amended) An expression vector comprising said nucleic acid molecule of Claim 9.